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METHODS FOR DETERMINING LIVE-DEAD ZEBRA MUSSELS (*DREISSENA POLYMORPHA*)

Report No 91-92-K

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ABSTRACT

Live veliger larvae are continuously motile and need only be microscopically examined to distinguish live from dead individuals. Movement of internal organs can be readily observed through the transparent shells in both veligers and post veligers, therefore live individuals can be easily recognized. Observation of shell movement, i.e. opening or closing is the simplest means of determining whether fully mature mussels are live or dead. Increased stress on adult *Dreissena polymorpha* increases the time the bivalve remains closed upon return to optimal conditions. Electrical stimulation offers the best means of inducing live mussels to open or close their valves under control (unstressed) or stressed conditions, such as exposure to chlorine. Exposure to thermal stress lengthens the time required for bivalves to open. Temporary immersion in water of increased salinity results in mortality in the 1.0 mm size class and lengthens the time required for larger bivalves to open.

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EXECUTIVE SUMMARY

METHODS FOR DETERMINING LIVE-DEAD ZEBRA MUSSELS (*Dreissena polymorpha*)

E.G. Leitch and B. Sim

The objective of this study was to identify efficient methods for determining whether veligers, post-veligers and adult mussels are live or dead following exposure to physical and chemical control treatments. As veligers are <350 µm in diameter, a microscopic technique was required to determine whether veliger larvae were live or dead. With juvenile (1.0 - 5.0 mm) and adult (> 5.0 mm) mussels, techniques were evaluated for their ability to induce movement only in mussels which were alive thereby distinguishing between live and dead individual mussels.

Live mussels respond to stress, handling, disturbance etc. by immediately closing their shells and will remain closed for at least as long as the stress continues. Whether or not a mussel control treatment, such as chlorine, has induced mortality cannot be determined in a closed mussel but it was expected that live mussels would open upon return to optimal conditions. Three techniques were selected to establish viability ; thermal-shock, electric-shock, and osmotic shock, all of which are known to elicit movement in the comparable marine mussel *Mytilus edulis*. The three treatments were evaluated against control groups of mussels which were microscopically observed after normal handling and no other additional stress. All sizes of mussels were used in the test and also mussels which had been exposed to sodium hypochlorite in typical station control concentrations. Thereby the results of the experiments could be directly applied to field situations.

The results demonstrated that as live veligers are highly motile, they need only be microscopically examined to determine live from dead larvae. With juvenile and adult mussels, regardless of mussel size, the time taken to open the shell valves from shortest to longest elapsed time, after removal of the various stress stimuli, was consistently in the following order: electric shock > normal handling > thermal shock > osmotic shock. The opening of the shell induced *via* electric stimulus was observed within 7 to 48 seconds (averaging approximately 22 seconds)

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after application of electric current. For normal handling, the equivalent times were 13 to 240 seconds (67 seconds on average). Post-thermal shock required 30 to 1800 seconds (249 seconds on average) before movement was observed and post-osmotic shock mussels took 510 to 2250 seconds, 1257 seconds on average.

Use of electric stimulus induced movement in mussels approximately three times more quickly than the next most efficient treatment which was normal handling. Electric shock was equally effective on chlorine exposed mussels. Both thermal and osmotic treatments resulted in response times, i.e., movement, many orders of magnitude greater than electric shock. The greater the stress, the longer the recovery time during which the mussels remained tightly closed, and completely immobile therefore live mussels could not be distinguished from dead mussels. Thermal and osmotic shock increased the recovery time upon return to optimal conditions before movement was observed as they induced greater stress than normal handling. Therefore, in the absence of electric shock apparatus, microscopic observation of movement in mussels which have not experienced stress other than unavoidable minimal handling is the most rapid means of determining live from dead mussels.

Electric shock is an efficient technique for the separation of live-dead mussels as it induces rapid movement in live mussels. Use of any other technique results in excessively stressing the mussels and subsequently increases the length of time the mussels remain closed thereby retarding the separation of live from dead mussels.

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To Mr. M.J. Northfield
Manager
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METHODS FOR DETERMINING LIVE-DEAD ZEBRA MUSSELS (*DREISSENA POLYMORPHA*)

1. INTRODUCTION

The recent introduction of the zebra mussel (*Dreissena polymorpha*) into the Great Lakes has resulted in a serious fouling problem for all utilities utilizing raw lake water. Although mussels may exert several significant effects when allowed to colonize pipes and support structures, the most rapid detrimental effect induced by zebra mussels is the obstruction of cooling water intakes and service water lines.

In response to the mussel invasion, Ontario Hydro initiated a research program to develop effective mussel control measures which would prevent colonization and subsequent power deratings within generating stations. Chlorination of service water systems is the only effective control option available at present. The possible concomitant effect of chlorine on non-target organisms may be environmentally unacceptable in the long term which could limit the use of chlorine to the immediate future only or to the introduction of dechlorination before discharge. Research is therefore directed towards chlorine minimization and the development of environmentally acceptable alternatives. These alternatives include various chemical and physical treatments (ozone, antifouling coatings, acoustics, gamma and UV irradiation, elevated pressure and temperature and mechanical filtration).

In order to evaluate any control treatment it must be possible to determine whether the mussels are alive or dead following the treatment. The objective of this project is to establish a method for distinguishing live from dead zebra mussels which is more rapid than conventional visual examination. The method must be reliable and accurate.

Although there are several available techniques for indicating whether molluscs are alive, e.g. monitoring respiration, the most rapid and easily executed technique is observation of movement of the mollusc. There are two types of easily observable movement in mussels: opening or closing of the shell valves, and, translocating across substrata by means of the muscular foot extended through the valves. Only detached mussels translocate, but all mussels, whether attached or detached must open the shell valves for physiological purposes.

Post-veliger, juvenile and adult zebra mussels are highly efficient filter-feeders and food is filtered from water drawn into the mussel *via* an inhalant siphon and ejected through an exhalant siphon. This process can only be conducted when the siphons are extended through the open valves. Under normal conditions, mussels normally respire aerobically and this process also requires a throughflow of oxygenated water from open valves into the mantle cavity and gills. Therefore under normal physiological conditions the mussels valves are open or gaping even if the siphons are not extended and movement of the gills etc. can be clearly observed if the mussels are not disturbed. Live mussels which are open at the time of examination can be very easily induced to close simply by prodding them (tactile stimuli). Mussels which are open (gaping) but are moribund or dead cannot be induced to close using tactile stimuli. Therefore tactile stimuli can be very effective in distinguishing live from dead mussels which are open at the time of examination. However, if exposed to any kind of disturbance or stress such as handling, thermal shock, chemical introduction, exposure to air, etc., the mussels will immediately close their shells. Live mussels can remain closed (as can dead mussels) for extended periods of time depending on ambient temperature and humidity. Therefore the difficulty with using simple observation of movement as a technique for distinguishing live from dead mussels is that live but closed mussels can stay completely stationary for significant periods of time when a rapid examination is required.

Therefore before any control treatments can be evaluated in the laboratory or field, a means of unequivocally determining live from dead mussels is required. As large numbers of organisms may be involved in post-control treatment mortality testing, a rapid technique is essential. Although observation of movement is relatively the most rapid means of distinguishing live from dead mussels it was necessary to identify techniques which would induce movement in live mussels, and in particular, opening in closed mussels.

Several techniques were selected and compared for effectiveness. The criteria for technique selection were: feasibility, rapidity of execution, reproducibility of results, and favourable responses elicited in the comparable marine mussel *Mytilus edulis*. *Mytilus*, like *Dreissena* will close during stress or handling and will remain closed for a variable period of time depending on ambient conditions. *Mytilus* are very sensitive to changes within their environment and not only will they close during immersion in unfavourable conditions but they also quickly reopen upon resubmergence into ideal conditions. For example, when subjected to more saline or warmer water than at acclimation, *Mytilus* close their valves but upon reimmersion into ideal water, open within a short space of time. The stress stimuli were not intended to induce the mussels to close, although shell closure invariably occurred, but rather, to briefly expose the mussels to sub-critical unfavourable conditions. After the removal of unfavourable conditions, the mussels would be encouraged to reopen and continue normal metabolism in optimal water.

The three techniques selected for comparison were brief exposure to thermal shock, osmotic shock and electrical shock. The results were compared against observed opening of valves in unstressed control groups of mussels.

2. METHODS AND MATERIALS

2.1 Laboratory Maintenance and Handling of *Dreissena polymorpha*

Mature mussels were collected from Lake Erie during May 1990 and returned to the laboratory. The mussels were cleaned and size sorted by washing them through a series of graded sieves. The mussels were then suspended in plastic mesh cages in aquaria containing dechlorinated tap water which was aerated and recirculated through a filtration pump. For most trials, mussels were maintained at 20 $\pm$1°C, for thermal shock trials some mussels were cold acclimated to 10°C. A photoperiod of 8:16 hours light:dark was established and the mussels fed every second day with dried *Chlorella* algae (Nutri-dyn Products Ltd., Alberta). Water quality was maintained at optimal level and replaced if necessary. Three sizes of mussels were selected: 1.0 mm, 5.0 mm and 10.0 mm in anterior-posterior axis length. Live, gaping and siphoning mussels were chosen for the tests.

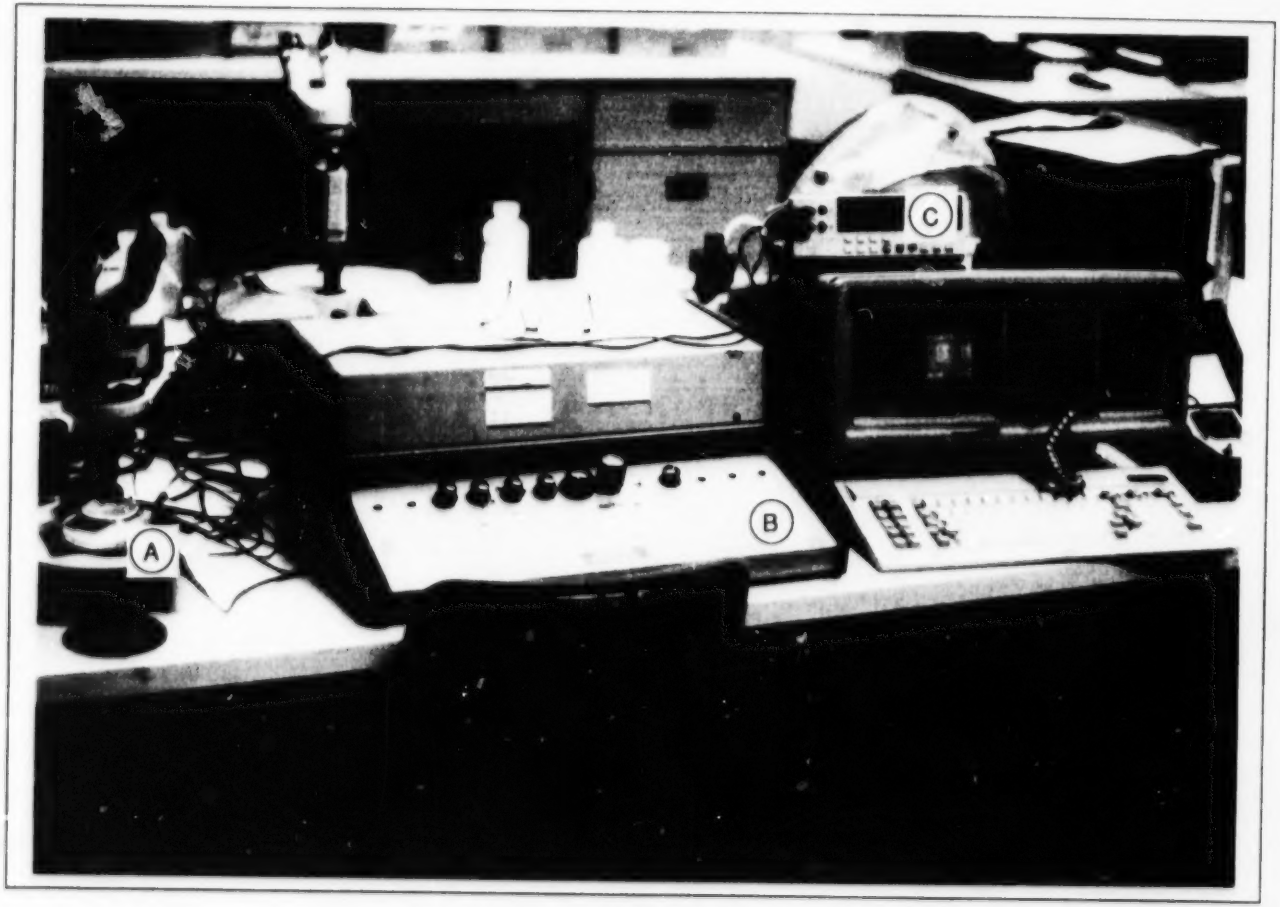
Examination of movement in the control groups of mussels in the laboratory involved transferring mussels from the acclimation aquaria to a water-filled petri-dish mounted on a binocular microscope stage. Although unavoidable, brief handling resulted in tactile stimuli which subsequently induced valve closure in the mussels. However, no other stresses were involved and the mussels were then microscopically observed for valve opening. The treatment and examination of the control group of mussels is referred to hereafter as "normal handling". All other groups of mussels would experience additional stress and would also undergo three times more handling than the control group. Therefore although the control group were not unstressed, they were minimally stressed when compared to all the other test groups. All mussels were only ever used once and after evaluation for movement (valve opening) were discarded to avoid accumulating stress.

Veligers were produced from laboratory cultures but remained viable for only 72 hours. Sub-samples of veligers were removed from holding chambers using sterile pasteur pipettes and examined at x200 magnification.

2.2 Live-Dead Determination Using Electrical Shock

2.2.1 Apparatus

The electrical shock apparatus (Figure 1) consisted of a high voltage function generator attached to the two electrodes of the electrode cell (Figure 2). The cell base was constructed of 'Perma-Base' denture acrylic into which the first electrode, a piece of stainless steel pipe 5.2 cm in diameter and 2.0 cm high, was embedded prior to hardening. A section of 2.0 mm stainless steel wire was the second electrode which was partially embedded in the centre of the base leaving 1.0 cm exposed. This arrangement ensured the two electrodes did not make contact. The cell was mounted to the base of a binocular microscope the field of vision being the entire cell. The cell was illuminated during observation of mussel behaviour.



(A) Test cell on microscope stage

(B) High voltage function generator

(C) Multimeter

FIGURE 1

ELECTRIC SHOCK APPARATUS

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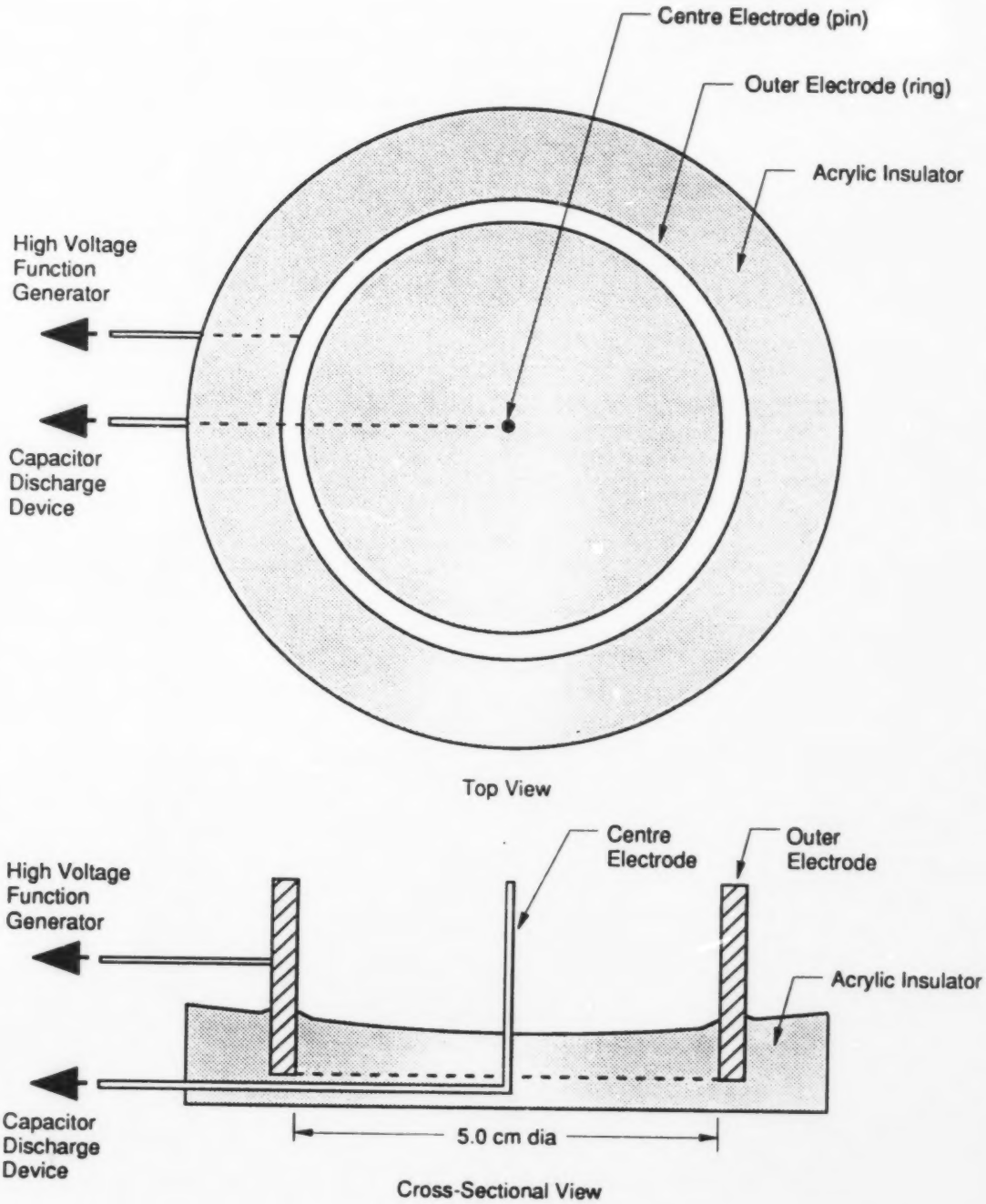

FIGURE 2

ELECTRODE CELL CONNECTED TO THE HIGH VOLTAGE FUNCTION GENERATOR OR CAPACITOR DISCHARGE DEVICE
(modified from McKinley, 1984)

The high voltage function generator was designed and constructed by Hydraulic Studies (Design and Development Division). The device is capable of producing three waveforms; sine, triangular and square and a variable voltage from 0-80 V rms through water with a conductivity of 355 $\mu S\ cm^{-1}$. Water conductivity was measured using a Canlab C500 conductivity meter. Amperage varied with voltage to a maximum of 0.06 A. Frequency ranged from 0 to 8000 Hz. Frequency and voltage (rms) were measured using a dual display multimeter (Fluke 45, Fluke Mfg. Co. Inc.; Everett, WA, USA).

An illustration of voltages and amperages within the electrode cell when coupled to the high voltage generator are shown in Figures 3 and 4. Measurements were made with across cell voltage of 18.1 V rms and 0.006 A at a fixed frequency of 1766 Hz and with water conductivity at 355 $\mu S\ cm^{-1}$.

In addition to using the high voltage function generator, a portable hand-held capacitor discharge device was also evaluated. This device was specifically designed and fabricated by Hydraulic Studies, Design and Development for determining live-dead zebra mussels using electrical shock. It differs from the high voltage function generator in that it produces a fixed DC voltage. The 6 volt DC power supply is stepped up to 200 volts DC which is used to charge a capacitor. The charged capacitor can then be discharged through the electrode cell. Details of this device are given in Appendix 1.

2.2.2 Method

Initial tests to determine the most efficient electrical shock regime were conducted using ten healthy zebra mussels from each size class. Mussels were removed from the holding tanks and individually positioned in dechlorinated water in the electrode cell along the spoke axis near the centre pin (Figure 3). The spoke axis is any radius between the central and the circumferential electrodes. The cell water was replaced regularly to prevent temperature increase within the cell due to the microscope light and the electric current.

Each individual was subjected to electrical shock using sine, triangular and square wave forms. Electrical shock, using each of these wave forms individually, was applied by first selecting an arbitrary frequency and then varying the voltage. Alternatively, the voltage was fixed and then the frequency was varied. The responses of each mussel were observed through the microscope during the procedure and recorded.

Subsequent tests using the most effective electrical parameters and method were conducted on twenty-five healthy mussels from each size class and the times required before movement was observed were recorded. The times required to observe movement were determined for a control group of mussels from each size class which were similarly placed in the electrode cell but not subjected to electrical stimulus.

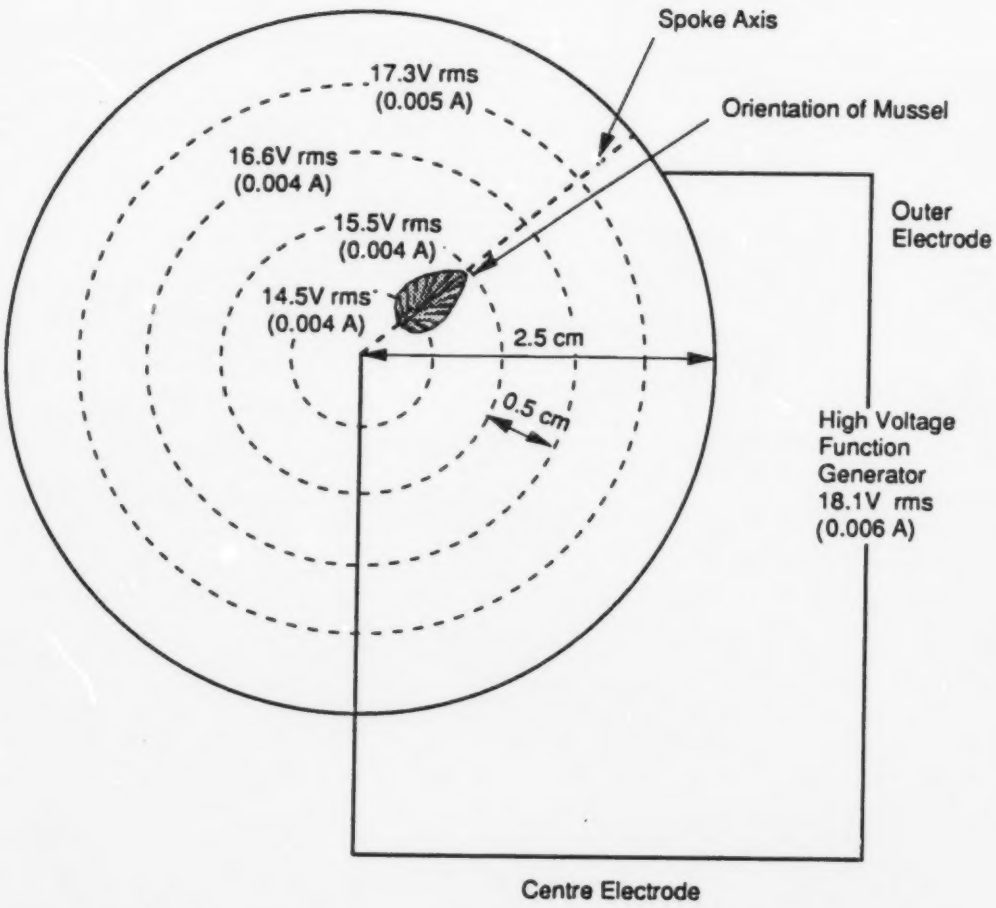

FIGURE 3

VOLTAGE AND AMPERAGE
MEASURED FROM THE CENTRE ELECTRODE
(Conductivity of 355 $\mu S\ cm^{-1}$)
(modified from McKinley, 1984)

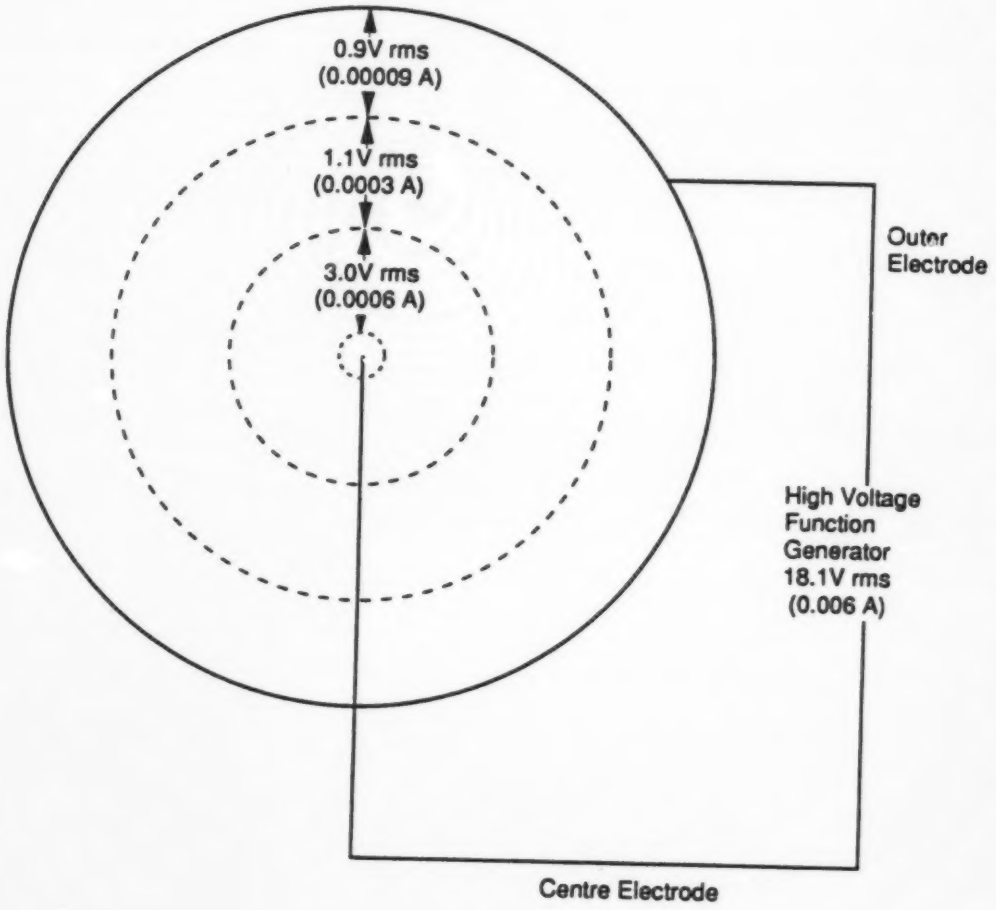

FIGURE 4

VOLTAGE DIFFERENCES AND ASSOCIATED CURRENTS BETWEEN TWO POINTS APPROXIMATELY 1 cm APART AT VARYING DISTANCES FROM THE CENTRE ELECTRODE
(Conductivity of 355 $\mu S\ cm^{-1}$)
(modified from McKinley, 1984)

The capacitor discharge device was tested by placing each individual mussel lengthwise along the spoke axis and discharging the stored charge through the cell. Ten individuals of each size class were tested.

2.3 Live-Dead Determination Using Thermal Shock

Mussels were subjected to various regimes to determine if any thermal pre-treatment could elicit valve opening upon reimmersion into water of ideal temperature. Treatments which were known to produce successful responses in marine mussels were selected. As any selected live-dead determination technique was required to be easily reproducible under field conditions, duration of treatments had to be minimized. Twenty-five mussels from the 5.0 mm size class were used in each treatment. For all treatments except B, mussels were acclimated at room temperature prior to being exposed to thermal shock for 10 minutes. Mussels for treatment B had been cold acclimated at 10°C. All mussels were exposed to thermal shock for 10 minutes and then transferred to room temperature for observation. The time required to determine movement of each mussel was then measured. Treatments were as follows:

Treatment	Acclimation Water Temp. (°C)	Thermal Shock Treatment (10 mins)	Treatment Stress	Observed Water Temp. (°C)
A	21	25°C (air)	+4°C temperature, dehydration	21
B	10	21°C (water)	+11°C temperature	21
C	21	21°C (air)	dehydration	21
D	21	25°C (water)	+4°C temperature	21
E	21	10°C (water)	-11°C temperature	21
Control	21	none	none	21

2.4 Live-Dead Determination Using Osmotic Shock

Subjecting the marine mussel *Mytilus edulis* to periods of immersion in fresh water or into super-saline water induces rapid closure of the valves. Upon reimmersion in seawater of normal salinity, *Mytilus* quickly open. By altering (increasing) the salinity of the water supporting zebra mussels, it was hoped that they would similarly respond upon reimmersion in fresh water. Forty mussels of each size class were immersed in full strength seawater (salinity = 35 ppt) at 21°C for various exposure periods of up to 60 minutes. The salinity of the synthetic seawater (Super Salt, Fritz Chemical Co., Dallas, Texas) was determined using a temperature compensating liquid

prism refractometer (Reichert-Jung, Cambridge Instruments Inc. Buffalo, NY, USA). Ten mussels from each size class were sequentially removed after 15, 30, 45 and 60 minutes, thoroughly rinsed with dechlorinated tap water, and transferred to ambient dechlorinated tap water. The time required for each mussel to open upon return to ambient conditions was recorded.

2.5 Blind Test Assessment

To eliminate operator bias, a blind test was conducted in which a mixed sample containing a known number of live and dead mussels was prepared by one individual and subsequently assessed by a second person. Mussels were killed for this trial by brief exposure to sodium hypochlorite. Of the three treatments evaluated for live-dead determination, the treatment which elicited a response in the shortest time period was used for blind live-dead assessment. Live-dead determinations were completed on triplicate samples (10 mussels per sample) for mussels of each size class. Mussels which were initially non-responsive were removed to a separate petri-dish and retested later. If they still remained unresponsive, they were then confirmed dead.

2.6 Chlorine Stress Assessment

The injection of sodium hypochlorite into the service water systems of utilities subjected to mussel impact is, at present, the only method available for effective mussel control. It is therefore necessary to determine if mussels which have been subjected to chlorine exposure can be induced to respond in a manner which will identify live from dead individuals. The test apparatus consisted of a 20 litre tank with a flow through water system.

Make up water was dechlorinated tap water in which a chlorine injection system was installed to maintain a residual chlorine level of 2.0 ± 0.18 ppm which mimics the intermittent treatment regime at Ontario Hydro facilities. The chlorine monitor (Capital Controls, Residual Analyzer/Monitor model 1870E) was adjusted to activate a Cole-Parmer "Unified" drive with a Masterflex pump head which introduced chlorine when the level fell below 2.0 ppm.

Fifty laboratory 21°C acclimated mussels of three size classes were transferred from the holding facilities and retained in plastic mesh baskets in the experimental tank, one basket for each size class. Sub-samples of ten individuals were assessed after 10, 20, 30, 60 and 180 minute exposure times using the treatment which elicited a live response in the shortest time period. Each sub-sample of ten individuals was rinsed in dechlorinated tap water after exposure to chlorinated water and transferred to fresh dechlorinated tap water before live-dead determinations were made.

3. RESULTS

3.1 Veliger Larvae

Live veligers are highly motile when viewed under a binocular microscope and require no further tests or manipulation to determine live from dead larvae. Even when the veligers are not rotating or travelling in typical orbital fashion, movement of the velum, cilia and internal organs is continuous and unmistakable in live larvae.

3.2 Electrical Shock

Preliminary tests using the high voltage function generator for the determination of the electrical parameters which would cause a response from a bivalve indicated that the sine wave (alternating current) was the most efficient. Direct current and triangular wave forms did not cause any observable response in the mussels. There was no specific set of parameters (voltage or frequency combination) that caused a mussel of any size class to respond. In general 1.0 mm mussels responded to 14.0 V rms and varied frequency. The 5.0 mm mussels responded best to 17.0 V rms and varied frequency. The 10.0 mm mussels responded to 18.0 V rms and varied frequency. (Appendices 2, 3 and 4) The mussels appeared to respond to specific frequencies and voltage but this was different for each individual mussel. Each mussel appeared to have a specific tolerance level to the applied electrical stimulus with respect to frequency and voltage.

In general mussels which were closed prior to the stimulus opened during electrical shock, and mussels which were open, closed. In some cases electrical stimulation would cause the siphons and/or the foot to protrude. With some combinations of frequency and voltage the bivalve could be induced to open and shut several times within a few seconds (clapping). Movement of the mussel tissue at the translucent anterior edge of the shell could be clearly observed during electrical stimulation.

Based on the results of the above preliminary tests, sine wave and fixed-frequency-varied-voltage and/or fixed-voltage-varied-frequency methods of operation, were applied to twenty-five mussels of each size class. The time required to observe movement is given in Appendices 5, 6 and 7 and Figures 5, 6 and 7 for 1.0 mm, 5.0 mm and 10.0 mm mussels respectively. Notched box plots were used to graphically present the data as they are convenient for determining differences between groups. The boxes are notched at the median and return to full width at the upper and lower confidence internal values. For an explanation of box plots, see Appendix 8. To be effective at inducing movement, the treatment had to induce a rapid response i.e. within 5 minutes and the percentage responsiveness of each size class of mussels to each treatment over time is given in Tables 1 to 3. From the tables it can be seen that for all sizes of mussels electric shock induced movement in mussels more quickly than movement was induced in the control groups (normal handling). Within 1 minute, 100% of the 1.0 mm mussels responded to electric shock compared to only 52% of the control group. With 5.0 mm mussels, again 100% responded within the first minute compared to 64% of the controls. However, only 88% of the 10.0 mm mussels responded to electric shock within 1 minute and 52% of the control group

responded within this time. However, although the data in the Tables indicate that electric shock induced movement in mussels in a shorter period of time than the controls, Figures 5, 6 and 7 show that there are no significant differences between times to respond using electric shock or unstressed control (normal handling) for any size of mussel. The time elapsed before mussels responded is significantly shorter when using electric shock or normal handling than it is for any thermal treatment (Figure 6) or osmotic treatment (Figures 5, 6, 7). The results presented in Figure 8 indicate that electric shock induces movement in mussels in a significantly shorter period of time (95% c.l.) than with the unstressed control groups for 1.0 mm and 5.0 mm mussels. However, for 10.0 mm mussels, there is no significant difference in response time between electric shocked and control groups of mussels. The range of response time is always shorter using electric shock than for unstressed controls for all mussel sizes.

Tests using the capacitor discharge device showed that electrical stimulation succeeded in closing mussels which were open but did not stimulate gaping in closed mussels. While the average response time for 1.0 mm mussels was 67 seconds using electric shock and 129 seconds for the control group (Table 4), Figure 9 shows that there was not a significant difference between the two groups of mussels. For the 5.0 mm and 10.0 mm mussels, the response times were statistically similar between electroshocked and unstressed control mussels.

3.3 Thermal Shock

Thermal shock was only tested using the 5.0 mm size class of mussel. The times taken for the mussels to respond to thermal shock are given in Figure 6 and Appendix 6.

The percentage responsiveness of the mussels to each of the five thermal shock treatments is given in Table 2. From the Table it can be seen that the order of thermal treatment which produced the most rapid response within the first 3 minutes was D>B>A>C>E. This is also the order of response presented in Figure 6. However, within 5 minutes of reimmersion in optimal temperature water , the responsiveness to treatment (i.e. observation of movement) was in the order: D>B>E>A>C. The order of response is slightly different between the data presented in Figure 6 and Table 2 because only the responses within the first 5 minutes of observation are considered in the Table whereas all the times to respond are presented in Figure 6. Overall, exposure to thermal treatment D produces a significantly more rapid response than all the other treatments and treatment B induces significantly quicker opening than treatment C. No other significant differences were observed between the treatments. Opening of mussels was most quickly observed with mussels which had been exposed to water of higher temperature than that at acclimation (D, B), followed by those exposed to water of lower temperature than at acclimation (E), and finally from those exposed to air (A, C). The range of response times was greater for treatments A and C than for the other treatments. The unstressed control group responded in a significantly shorter period of time than the mussels exposed to any of the thermal treatments.

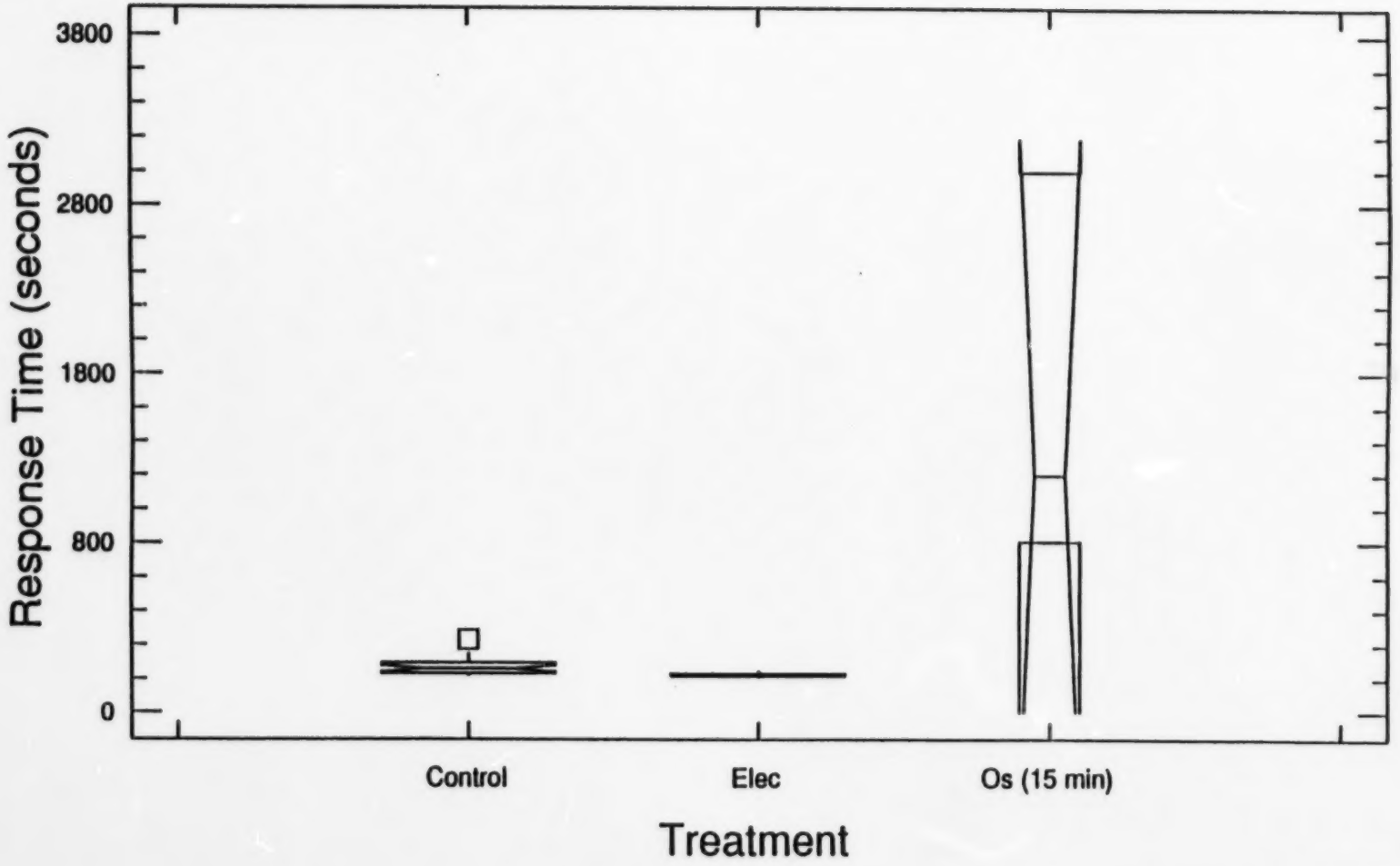

Figure 5 Response time of 1.0 mm mussels subjected to electric shock, osmotic shock and control (normal handling)

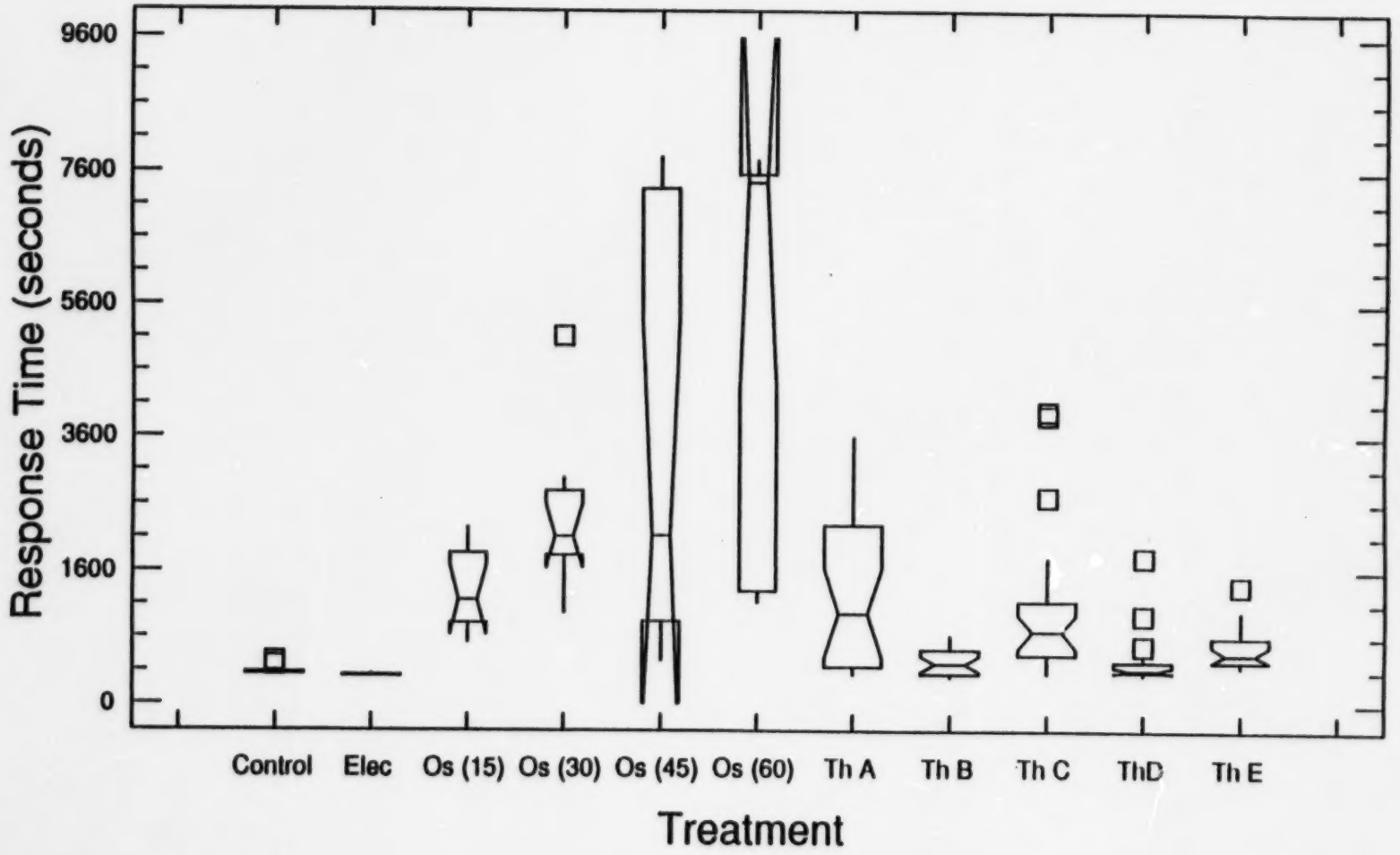

Figure 6 Response time of 5.0 mm mussels subjected to electric shock, osmotic shock, thermal shock and control (normal handling)

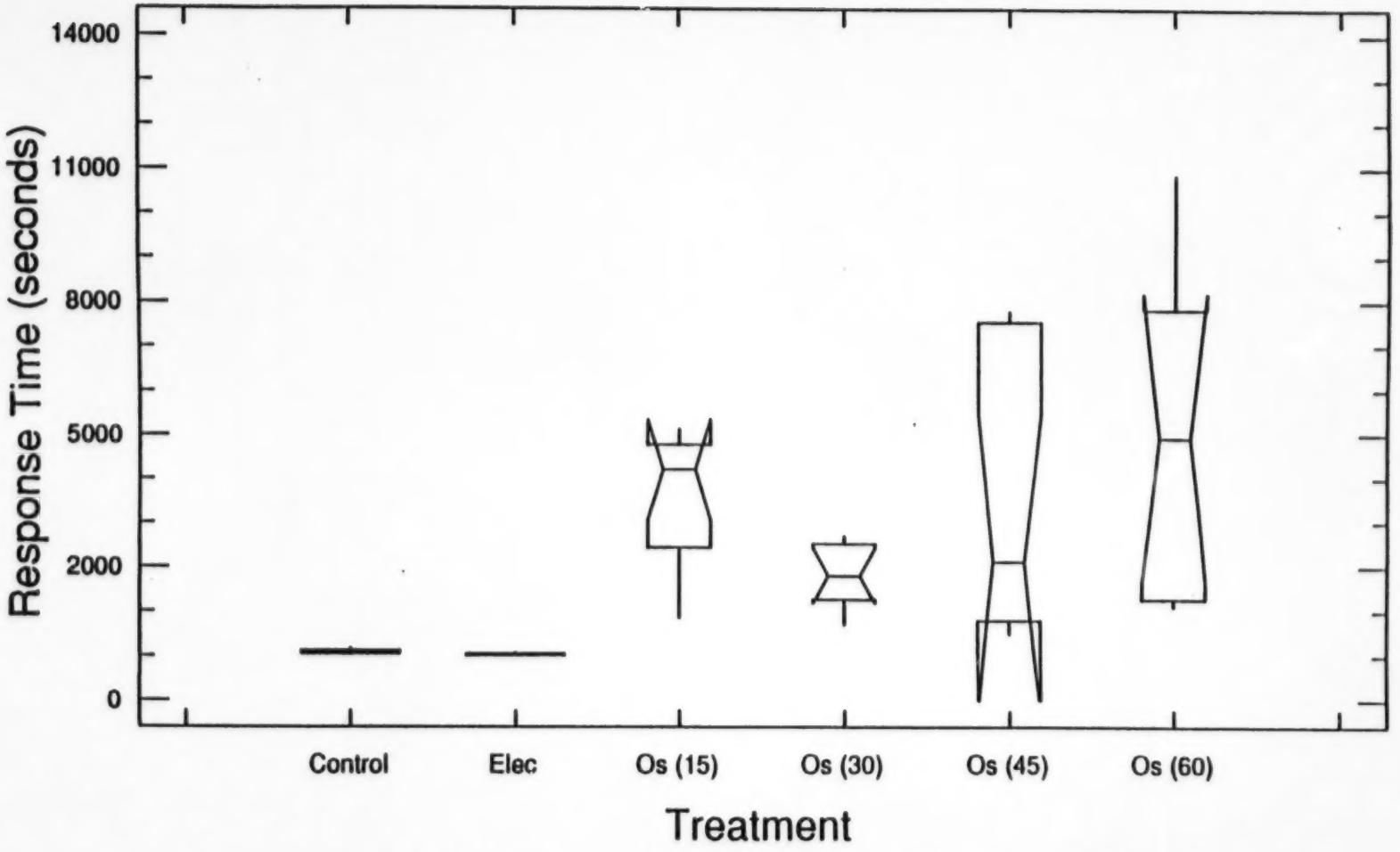

Figure 7 Response time of 10.0 mm mussels subjected to electric shock, osmotic shock and control (normal handling)

Table 1 Percentage responsiveness of 1.0 mm mussels to treatment over time.

Time (min) / Treatment		1	3	5
Electric shock		100	100	100
Osmotic Shock	15 min	0	0	0
	30 min	0	0	0
	45 min	0	0	0
	60 min	0	0	0
Control (normal handling)		52	96	100

Table 2 Percentage responsiveness of 5.0 mm mussels to treatment over time.

Time (min) / Treatment		1	3	5
Electric shock		100	100	100
Osmotic shock	15 min	0	0	0
	30 min	0	0	0
	45 min	0	0	10
	60 min	0	0	0
Thermal shock	A	20	28	36
	B	36	50	60
	C	4	12	24
	D	24	72	88
	E	0	8	44
Control (normal handling)		64	96	100

Table 3 Percentage responsiveness of 10.0 mm mussels to treatment over time.

Time (min) / Treatment		1	3	5
Electric shock		88	100	100
Osmotic shock	15 min	0	0	0
	30 min	0	0	0
	45 min	0	0	0
	60 min	0	0	0
Control (normal handling)		52	100	100

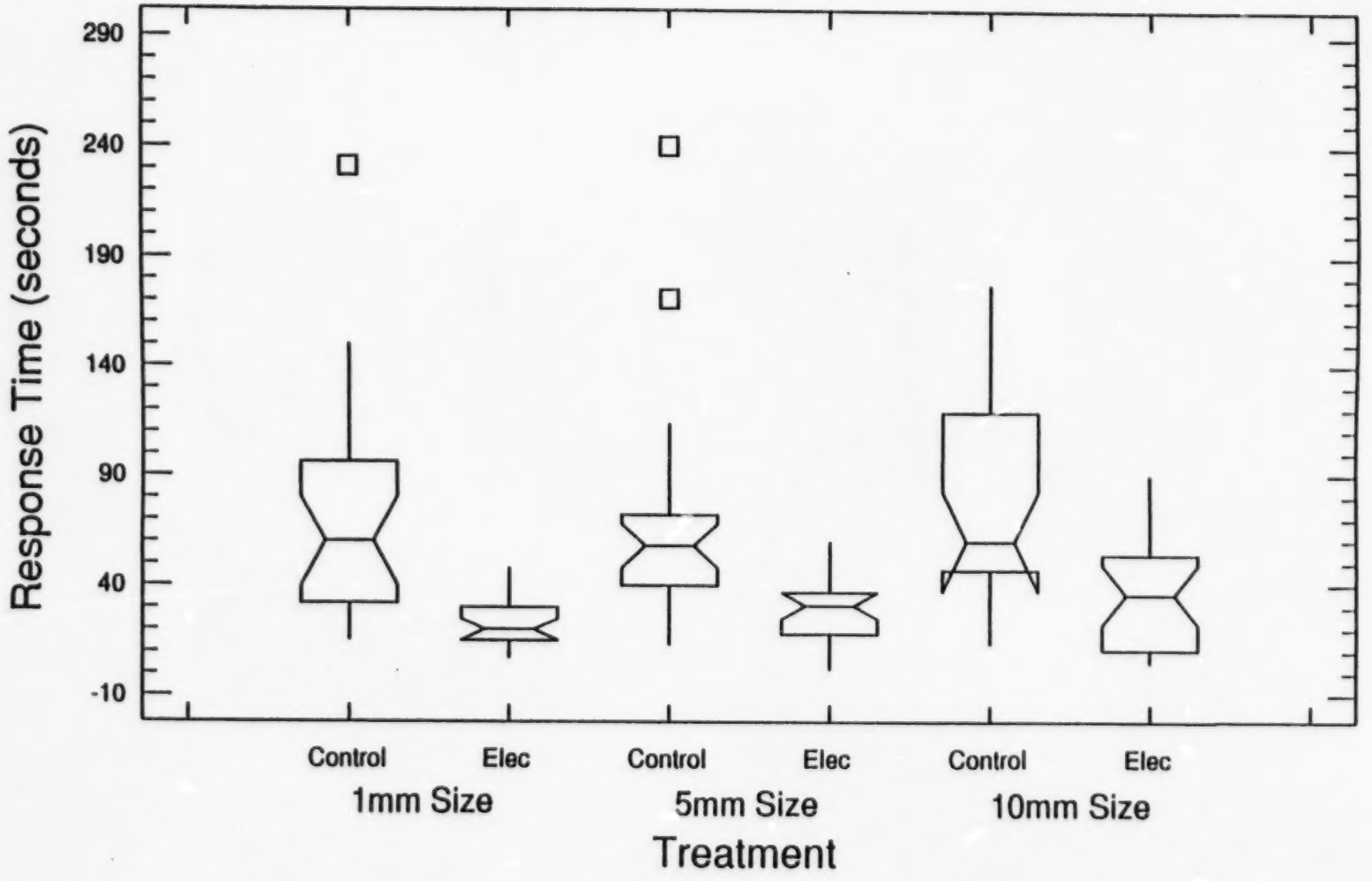

Figure 8 Response time of mussels subjected to electric shock and control (normal handling)

Table 4 Response times of mussels subjected to capacitor discharge electric shock and control (normal handling).

Size (mm)	1.0		5.0		10.0	
Specimen	Control (Normal handling)	Electric Shock	Control (Normal handling)	Electric Shock	Control (Normal handling)	Electric Shock
1	30	26	34	33	29	13
2	32	30	43	60	30	30
3	45	33	44	60	30	60
4	60	38	50	67	63	63
5	120	38	60	80	79	79
6	163	47	60	97	84	84
7	193	53	80	107	93	97
8	200	109	87	120	100	115
9	203	120	96	120	180	120
10	240	180	125	180	180	180
Mean	129	67	68	92	86	84
Std.	77	49	27	40	54	45

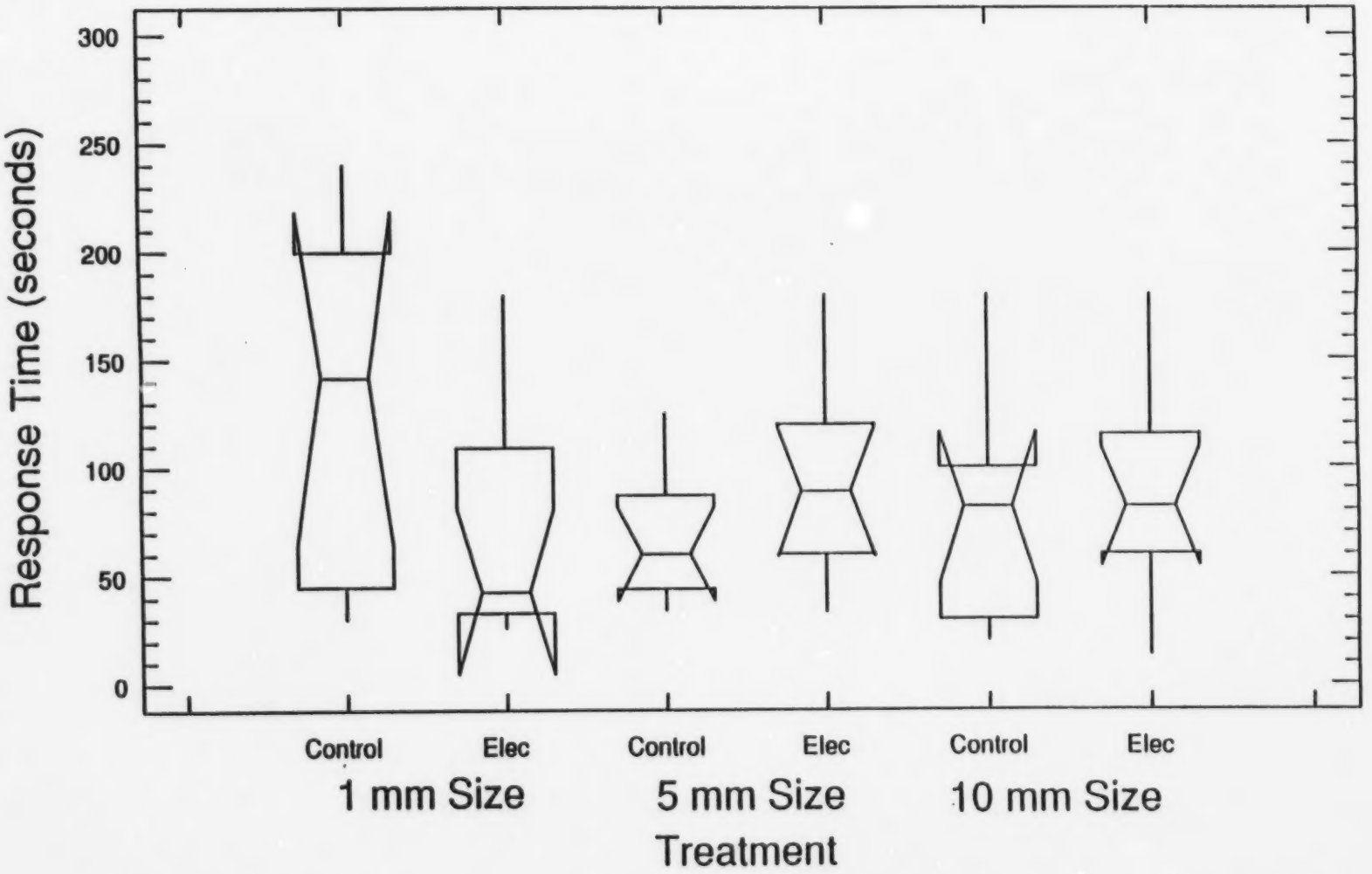

Figure 9 Response time of 1.0 mm, 5.0 mm and 10.0 mm mussels subjected to capacitor discharge electric shock and control (normal handling)

3.4 Osmotic Shock

The response times for the three size classes of mussels subjected to osmotic shock for varying exposure periods from 15 to 60 minutes are given in Figures 5, 6 and 7 and Appendices 5, 6 and 7. In the 1.0 mm size class, only three of the ten mussels survived 15 minutes exposure to seawater. Upon return to optimal water conditions the three mussels did not open for 810, 1200 and 3000 seconds respectively. When the exposure time in seawater was increased beyond 15 minutes, no 1.0 mm mussels survived.

In the 5.0 mm size class there were no mortalities within the 60 minute exposure period. In general, the response time increased as the exposure time in seawater increased. The post-osmotic shock treatment recovery times invariably required time in excess of 5 minutes before movement was observed after most of the exposure times (Tables 1 to 3). Only 10% of the mussels responded within 5 minutes after exposure to water of increased salinity for 45 minutes. All other groups failed to respond within this time. From Figure 6, exposure to osmotic shock for 15 minutes resulted in a significantly more rapid response time than exposure for 30 minutes. No other significant differences were observed. The range of response times after 15 and 30 minute exposures was very much shorter than for treatments of 45 and 60 minutes. Unstressed control 5.0 mm mussels opened in a significantly shorter time than any of the mussels exposed to osmotic treatment.

For the 10.0 mm mussels, no movement was observed within 5 minutes after return to water of optimal salinity regardless of the duration in seawater (Table 3). Mussels exposed for 30 minutes responded significantly more quickly than those exposed for 15 minutes and the range of response times was shorter for the 30 minute and 15 minute exposure period than for 45 and 60 minute exposures.

3.5 Comparison of Treatments

In comparing the average response times that the various treatments induced (Table 5) , it can be seen that the length of elapsed time before the mussels moved differed greatly depending on the treatment to which they had been subjected. Mussels which were electroshocked moved within a shorter time period than mussels which had undergone only normal handling (controls) which in turn recovered and opened more quickly than mussels after thermal treatment. Finally, the mussels which were recovering from osmotic shock required the longest time to open upon removal of the stress and return to optimal conditions. Time to respond (movement) is therefore in the following order: electric shock> normal handling (control) > thermal shock > osmotic shock. For all mussel size classes, movement induced *via* electric stimulus resulted in response times which were, on average, approximately three times faster than the treatment which induced the next most rapid response time, normal handing. Regardless of mussel size electric shock treatment always resulted in a shorter range of response time compared to any other stimulus. (Figures, 5, 6, 7). The range of response times for the minimally stressed control groups was also small. Much larger ranges of response times were observed for thermal and osmotic treatments. In the absence of electric shock apparatus, observation of movement in unstressed

Table 5 Comparison of treatments and response times

Size Class of Mussels																						
Treatment	1.0 mm						5.0 mm										10.0 mm					
	Electric Shock		Osmotic Shock Duration				Electric Shock		Osmotic Shock Duration				Thermal Shock Treatment					Electric Shock		Osmotic Shock Duration			
Response Time	Control	Stressed	15 min	30 min	45 min	60 min	Control	Stressed	15 min	30 min	45 min	60 min	A	B	C	D	E	Control	Stressed	15 min	30 min	45 min	60 min
Minimum (sec)	15	7	810	N/R	N/R	N/R	13	2	510	960	240	1110	30	10	60	30	150	14	5	840	960	410	1110
Maximum (sec)	231	48	3000	N/R	N/R	N/R	240	60	2250	5120	7830	7770	3610	630	3990	1800	1380	177	90	5760	2700	7800	10860
Mean (sec)	69	22	501	N/R	N/R	N/R	67	29	1257	2396	3780	5214	1344	251	1125	249	503	76	34	3879	1788	3813	5292

N/R = No response

mussels after minimal handling is the most rapid means of determining live from dead mussels of any size.

3.6 Blind Test Assessment

Of the three candidate live-dead determination techniques, the electrical shock treatment was selected for the blind test assessment since it elicited the most rapid response time in all size classes of mussels. The blind test data are given in Table 6. In all but one replicate in the 1.0 mm size class, live-dead separation of blind versus actual was exactly the same. In one of the 1.0 mm replicates , two mussels were assumed to be dead due to lack of response to electroshock but later observation confirmed movement. Electroshock did not kill any of the mussels and no mussels which were judged to be alive using electroshock were later found to be dead. Involuntary movement cannot be induced in dead mussels using the electric shock device in the manner described.

Table 6 Blind Test Evaluation: Live-Dead Determination Using Electric Shock

Size	Replicate	Number of Mussels	Electric Shock (Blind)		Control**	
			Live	Dead	Live	Dead
	1	10	2	8	4	6
1.0 mm	. 2	10	8	2	8	2
	3	10	9	1*	10	0
	1	10	2	8	2	8
5.0 mm	2	10	9	1	9	1
	3	10	6	4	6	4
	1	10	8	2	8	2
10.0 mm	2	10	8	2	8	2
	3	10	10	0	10	0

* crushed when transferred to cell
** Control - Predetermined by visual observation

3.7 Chlorine Stress Assessment

Of the three candidate live-dead determination techniques, the electrical shock treatment was selected for the chlorine stress assessment since it elicited the most rapid response time in all size classes of mussels. The objective of this test was to determine if chlorine stressed mussels would respond to the electrical stimulation, and all mussels tested after chlorine exposure responded to electroshock. Continuous exposure to 2.0 ppm chlorine for up to 180 minutes resulted in no mortality of mussels in any of the size classes tested.

4. DISCUSSION

All physiological stresses which can be detected by the mussel, whether physical, chemical or electrical result in an immediate closure of the valves. The objective of this research was to subject mussels to stress which would cause them to close but which would encourage rapid opening upon return to optimal conditions thereby indicating whether the mussels were live or dead. Laboratory acclimated mussels respond to various stress stimuli with respect to length of time required to recover and open their valves.

In comparing the response time of the different size classes of mussels to the same stimulus, the smallest mussels generally recovered more quickly on average than all other sizes when subjected to stressful stimuli. Observations of laboratory mussels have revealed that the smallest mussels are not only more motile than more mature mussels but they are also more vulnerable to changes in water quality, conductivity, temperature and handling. The small mussels may be more active because under natural conditions they must actively seek settlement and attachment sites by translocating across potential substrate surfaces prior to permanent attachment *via* the secretion of byssal threads. Small mussels possess much thinner shells than older, larger individuals and it is therefore beneficial for young mussels to have a heightened sensitivity to the environment prior to attachment. This increased sensitivity may explain why the 1.0 mm size mussels responded more rapidly to reimmersion in water of optimal quality.

In comparing the recovery times of mussels in any one size class when subjected to different stimuli, electric shock induced the most rapid response followed by normal handling (i.e., transferring the mussels from aquaria for microscopic observation in a water-filled petri-dish), thermal stress and finally osmotic shock. This sequence of temporal response was consistent regardless of the size of the mussels tested. Mussels which were closed when placed into the electrode cell opened during the application of electric current and those which were open prior to current application could be induced to close. This behaviour is the typical physiological response of muscle tissue which contracts during electrical stimulation by the nervous system. No other type of induced stress is expected to cause movement in mussels in this fashion.

In comparing osmotic stress with thermal stress and normal handling, the latter seemed to cause the least amount of stress. The mussels typically re-opened within 75 seconds and started to translocate. Having evolved from the marine environment *Dreissena* can withstand intermittent exposure to air such as occurs with intertidal invertebrates during low tide. Therefore, the brief

exposure to air experienced between transfer from aquaria to petri-dish for observation induces minimal stress and does not result in extended periods of valve closure.

Thermal stress resulted in much more prolonged periods of closure compared to normal handling even after return to cold water. The greater the degree of thermal stress, the longer the recovery time. Mussels acclimated to room temperature water then transferred to 25°C water for 10 minutes followed by return to ambient resulted in the most rapid recovery from thermal stress although it required 3-4 times greater than the recovery time after the least thermal stress treatment and 20 times greater than normal handling. In the natural environment mussels can experience thermal stress during summer if they inhabit small, shallow lakes or when attached near the air/water interface. *Dreissena* can survive only limited periods of exposure to elevated temperature, e.g., at 35°C, 50% morality will occur after 180 minutes but at 36°C, 50% mortality is observed after 100 minutes (Griffiths, 1990).

Exposure to osmotic stress resulted in the longest elapsed time before recovery after return to water of normal salinity. Recovery time after exposure to increased salinity was between 5 and 42 times longer compared to all other treatments. The mussels were severely stressed by even brief immersion in water of increased salinity and responded by closing for a long period of time. In the natural environment, *Dreissena* is mainly confined to freshwater lakes and rivers but some populations exist in slightly brackish estuarine areas. The retention of a flexible osmoregulatory capability by *Dreissena* ensures that this species can successfully colonize and exploit habitats of variable salinity. Typically most freshwater has a salinity of 0.1 to 0.3 ppt, with many brackish waters ranging from 0.3 - 20.0 ppt. The mussels used in this test were collected and maintained in water at ca. 0.3 ppt and had no previous exposure to increased salinity. In summarizing the European literature it would appear that the salinity tolerances of *Dreissena* are quite variable but are frequently at highest density in areas with mean salinities of <0.3 ppt although they have been reported surviving maximum levels of 6.76 ppt (MacNeill, 1990).

These results appear to suggest that *Dreissena* can close its shell to varying degrees depending on the stress level. Although *Dreissena* close when handled, this disturbance does not appear to cause much stress and they quickly recover. However, the more acute physiological stress caused by temperature increase or by salinity increase results in much more prolonged periods of closure before recovery. It is also likely that *Dreissena* are unable to sense the quality of the environment through the closed shell and they need to gape slightly to analyze external water chemistry and temperature. More detailed investigations into the mechanisms involved in the sensory capabilities of zebra mussels through closed shell valves are required.

During the blind test evaluation, two 1.0 mm mussels initially failed to respond to electric shock but later became mobile. It is most likely that this delay in response was due to handling which may have caused significant damage sufficient to render them immobile even during electroshock. This observation was recorded once and only with the smallest size of mussels. However, subsequently, not only was great care taken during handling of small mussels in order to minimize injury but small non-responsive individuals were always retested after a latent period.

The use of the electroshock device for live-dead determination during other experiments to determine the thermal and pressure tolerance of mussels also indicted the necessity of further precautions to avoid mis-identification of live-dead mussels. It was observed that air which was trapped inside dead bivalves could expand during electroshock application and the resultant movement could lead to false recording of live mussels. (Checko, E. pers comm., 1990). A dead mussel can be double-checked for movement by prying open the valves and again administering electric shock. The mussel tissue and foot will move or twitch if the mussel is alive even if moribund and in a condition of no recovery. There is absolutely no response if the mussel is dead. For critical determinations where initial live-dead results are inconclusive, electric shock should be reapplied after a delayed period. In addition, re-orientation of the mussel in the electrode cell should be undertaken. These modifications to the electroshock technique will produce the most reliable live-dead separation and minimize the likelihood of error. (See Appendix 9).

The experiment which tested the use of the electric shock device to determine live from dead mussels after exposure to chlorine indicated that this technique is equally effective for mussels exposed to 2.0 ppm total residual chlorine. By this means, it will be possible to rapidly check effectiveness of chlorination regimes on both juvenile and adult mussels. In addition to sodium hypochlorite, various alternative mussel control treatments, i.e. thermal shock, acoustics, high pressure, were also investigated in the laboratory. With each of these experiments the mussels were checked for post-experiment mortality using electric shock. These treatments did not apparently preclude a rapid response to electrical shock as a time-saving method for distinguishing live from dead zebra mussels.

5. CONCLUSIONS

1. When subjecting mussels of three size ranges to the stress stimuli, the 1.0 mm and 5.0 mm mussels recovered and opened in a significantly shorter period of time than the 10.0 mm size of mussels. Regardless of mussel size, electric shock induced movement in mussels in the shortest period of time when compared to thermal shock, osmotic shock or control (normal handling).

2. Of the three treatments tested, electric shock was the most effective in determining live from dead mussels. Mussels which were open could be made to close during electrical stimulus and mussels with the shell valves closed could be induced to open.

3. For all sizes of mussels the average recovery time using electric shock was approximately 28 seconds, and 71, 249 and 1,182 seconds for normal handling, thermal shock and osmotic shock respectively.

4. Using electric shock the most rapid and accurate results were obtained by rechecking undamaged but non-responsive mussels after a latent period of time, or, by prying open the valves and re-administering electric shock.

5. The greater the stress, the longer the recovery time during which the mussels remained tightly closed. Thermal and osmotic shock increased the recovery time as they induced greater stress than microscopic observation after normal handling.

6. The data collected in this study suggest that the mechanisms and sensory capabilities of zebra mussels through closed shell valves are not highly developed.

7. In the absence of electric shock, the best technique for live-dead determination is normal handling followed by unstressed microscopic examination. Use of any other technique results in stressing the mussels and subsequently increases the recovery time.

8. Use of mussels treated by other methods e.g. chlorination, thermal backwash etc. does not appear to affect the effectiveness of electroshock as a method of determining live from dead mussels.

6. RECOMMENDATIONS

1. Use an electric shock device wherever possible to determine live from dead mussles and if this device is not available, use microscopic observation after minimal handling.

2. If using electric shock, all 1.0 mm mussels which do not initially respond should be retested after a latent period.

3. Minimize handling of mussels during passive observation.

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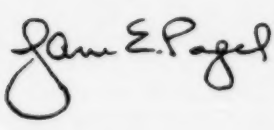

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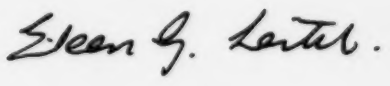

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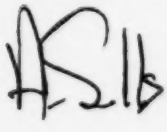

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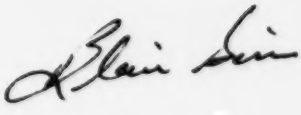

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Appendix 1

A High Voltage Function Generator
for the Determination o Live/Dead Zebra Mussels

SPECIFICATIONS

Frequency Range	5 Hz to 12 kHz
Output Voltage (Max)	500 V P/P
Waveforms	Sine, triangular, pulse
Distortion	2% (Max)
Isolation	Optical and transformer
Output Characteristics	Balanced
Output Current Limiting	24 ohm in series with 0.47 uF in each output phase
Battery	YUASA NP15-12 12 V 15 AH
Main Fuse	5 A

Circuit diagrams can be obtained from the author

DANGER THIS INNOCENT LOOKING 12 V BATTERY IS CAPABLE OF PRODUCING 500 V PEAK TO PEAK, AT A CURRENT OF 25 mA, AND IS THEREFORE POTENTIALLY LETHAL. ONLY TRAINED PERSONNEL SHOULD USE THIS APPARATUS.

Operating Procedure

1. Turn the amplitude potentiometer to zero.

2. Switch the power toggle switch to the OFF position (down).

3. Select the desired waveform, (use the three position rotary switch).

4. Connect the test cell to the output junction box. ENSURE THAT THERE IS NO CONDUCTIVE PATH BETWEEN THE TEST CELL AND YOU. i.e. WATER, METAL, AND EVEN WATERLOGGED WOOD CAN PROVIDE CONDUCTIVE PATHS.

5. Although the HVFG front panel controls and batteries are electrically isolated from the high voltage output, it is strongly suggested that rubber be worm when working with specimens in the test cell. THIS DOES NOT MEAN THAT PART OF THE OPERATING PROCEDURE CAN BE BYPASSED. Switch the power toggle switch ON.

6. Set the frequency of the HVFG by adjusting the front panel potentiometer, while simultaneously monitoring the frequency of the LCD display, or the FLUKE 45.

7. Monitor the RMS output voltage by using the front panel LCD display or the second display on the FLUKE 45.

8. Slowly increase the output level to the desired value.

9. Change the output waveform if desired, note the triangular and pulse waveforms have different spectral densities, and therefore will produce different RMS voltage outputs.

10. The waveform symmetry can be controlled by the front panel potentiometer.

11. Do not use the battery charger while simultaneously operating the unit in the field, unless a ground fault interrupter is connected between the 115 VAC supply and the battery charger.

12. The differential output from the HVFG is connected to a junction box which also contains two 0.47 uF capacitors and two 2k ohm resistors for DC isolation and output current limiting.

13. The impedance of the test cell can be determined by the voltage drop across the dual series isolation components, thus the cell voltage gradient can be calculated.

14. Turn the power toggle switch to the OFF position in between tests to conserve battery power. (The 12 V battery current is approximately 4A).

15. The LCD display is powered by a 9 V battery, (Ever Ready No 522). This battery should be replaced every six months.

G.A. Hunt
Design & Development Div
January 2, 1991

Appendix 2

Responses of 1.0 mm Zebra Mussels to Electrical Stimulation

Specimen	Voltage (V)	Frequency (Hz)	Initial condition of Mussel	Response to Stimulus
1	14.0	1700 - 7300	closed	opened
2	14.0	1700 - 7300	partially open	increased opening
3	14.0	1700 - 7000	closed	opened
4	14.0	1700 - 8000	closed	opened & closed (clapping)
5	14.0	550 - 3800	partially open	extended foot, became mobile increased opening
6	14.0	1700 - 6800	open	opened, gills exposed
7	14.0	1700 - 7900	open	closed
8	14.0	1700 - 7100	open	opened, extended foot
10	14.0	1700 - 7200	closed	opened

Appendix 3

Responses of 5.0 mm Zebra Mussels to Electrical Stimulation

Specimen	Voltage (V)	Frequency (Hz)	Initial condition of Mussel	Response to Stimulus
1	17	2275 - 4230	closed	opened
2	17	3800	closed	opened
3	17	2279	closed	opened
4	17	3917	closed	opened
5	17	550	partially open	increased opening and clapping
6	17	4030	closed	opened, extended siphons
7	17	4190	closed	opened
8	17	4300	closed	brief spasm
9	17	3100	closed	opened, extended siphons
10	17	1700 - 2100	closed	opened, extended siphons

Appendix 4

Responses of 10.0 mm Zebra Mussels to Electrical Stimulation

Specimen	Voltage (V)	Frequency (Hz)	Initial condition of Mussel	Response to Stimulus
1	18	5830	closed	opened
2	18	586	closed	opened
3	18	1590	closed	opened
4	18	1500	partially open	closed
5	18	1500	partially open	further opened and clapping
6	18	2000-4300	partially open	closed, clapping by varying voltage
7	18	1700-4460	partially open	closed, clapping by varying voltage
8	18	1800-3000	partially open	closed
9	18	1700-2400	partially open	closed
10	18	1700-2800	closed	opened

Appendix 5

Response Time of 1.0 mm Zebra Mussels Subjected to Osmotic Shock, Electric Shock and Control (Normal Handling)

	Response Time (sec)					
			Osmotic Shock			
Specimen	Normal Handling	Electric Shock	15 min	30 min	45 min	60 min
1	15	7	810	D	D	D
2	17	7	1200	D	D	D
3	18	8	3000	D	D	D
4	20	9	D	D	D	D
5	28	11	D	D	D	D
6	31	14	D	D	D	D
7	32	15	D	D	D	D
8	32	15	D	D	D	D
9	35	15	D	D	D	D
10	43	16	D	D	D	D
11	46	17				
12	50	20				
13	60	20				
14	65	21				
15	70	21				
16	74	24				
17	85	25				
18	90	29				
19	96	30				
20	99	33				
21	101	33				
22	112	35				
23	122	36				
24	150	40				
25	231	48				
mean	69	22	501			
STD	49	11	954			

D = treatment resulted in death of mussel

Appendix 6

Response Time of 5.0 mm Zebra Mussels Subjected to Electric Shock, Thermal Shock, Osmotic Shock and Control (Normal Handling)

Specimen	Response Time (sec)										
	Normal Handling	Electric Shock	Thermal Treatment					Osmotic Shock			
			A	B	C	D	E	15 min	30 min	45 min	60 min
1	13	2	30	10	60	30	150	510	960	240	1110
2	16	6	30	30	180	30	180	810	1440	360	1290
3	16	10	30	30	180	30	210	810	1830	840	1290
4	36	14	30	60	210	30	210	630	1830	2100	2160
5	38	15	60	60	240	30	226	1110	1830	2130	7410
6	39	15	90	60	288	60	234	1200	2400	2130	7470
7	40	18	160	60	342	90	240	1230	2760	7230	7500
8	40	19	240	60	390	90	240	1860	2790	7350	7560
9	43	20	240	60	468	90	240	2160	3000	7590	7680
10	52	20	360	90	600	120	300	2250	5120	7830	7770
11	52	27	840	120	630	120	300				
12	54	28	840	150	675	120	330				
13	58	31	960	210	720	120	360				
14	59	32	960	210	750	120	450				
15	60	32	1800	240	766	120	450				
16	60	32	1920	342	780	180	510				
17	61	33	2040	360	960	180	540				
18	66	35	2160	360	960	180	570				
19	72	37	2280	420	1140	240	600				
20	80	41	2430	420	1530	240	600				
21	87	42	2700	480	1800	240	900				
22	100	45	2910	480	2700	300	900				
23	114	60	3270	510	2700	480	960				
24	171	60	3600	570	3930	930	990				
25	240	60	3610	630	3990	1800	1380				
mean	67	29	1344	241	1080	239	483	1257	2396	3780	5214
STD	48	16	1228	193	1091	368	312	595	1096	3110	2898

Appendix 7

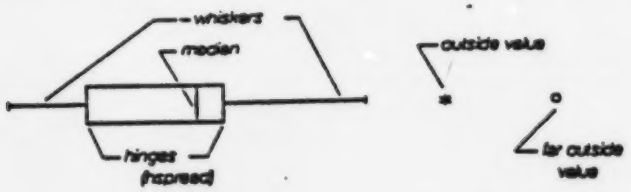
Response Time of 10.0 mm Zebra Mussels Subjected to Osmotic Shock, Electric Shock and Control (Normal Handling)

Specimen	Response Time (sec)					
	Normal Handling	Electric Shock	Osmotic Shock			
			15 min	30 min	45 min	60 min
1	14	5	840	960	510	1110
2	31	6	2400	960	810	1290
3	35	8	2430	1260	810	1290
4	37	8	3600	1560	1590	2250
5	38	8	3900	1800	2100	2280
6	41	10	4500	1800	2160	7590
7	47	11	4830	1800	7200	7620
8	47	11	5130	2520	7560	7830
9	49	12	5400	2520	7590	10800
10	52	16	5760	2700	7800	10860
11	54	21				
12	54	27				
13	60	36				
14	62	37				
15	69	39				
16	70	42				
17	75	43				
18	83	46				
19	119	54				
20	124	59				
21	127	60				
22	130	60				
23	149	64				
24	160	78				
25	177	90				
mean	76	34	3879	1788	3813	5292
STD	44	24	1494	600	3086	3825

APPENDIX 8

Box Plot Interpretation*

The median of the batch is marked by the center vertical line. The lower and upper hinges comprise the edges of the central box. The median splits the ordered batch of numbers in half, and the hinges split the remaining halves in half again.



Definitions

To understand the remainder of the box plot, we need some definitions. *Hspread* is comparable to the interquartile range or midrange. It is the absolute value of the difference between the values of the two hinges.

The *inner fences* are defined as follows:

lower fence = lower hinge – (1.5Hspread)
upper fence = upper hinge + (1.5Hspread)

The *outer fences* are defined as follows:

lower fence = lower hinge – (3Hspread)
upper fence = upper hinge + (3Hspread)

The *whiskers* show the range of values which fall within 1.5 Hspreads of the hinges. The do *not* necessarily extend to the inner fences.

Values outside the inner fences are plotted with asterisks. Values outside the outer fences are plotted with empty circles.

* Wilkinson, Leland. SYGRAPH: The System for Graphics. Evanston, Il: SYSTAT, Inc., 1990.

McGill, R., J.W. Tukey and W.A. Larsen. 1978. Variations of Box Plots. The American Statistician, 32:12 - 16.

Notched box plots

McGill, Tukey, and Larsen (1978) implemented confidence intervals on the median of several groups in a box plot. If the intervals around two medians do not overlap, you can be confident at about the 95% level that the two population medians are different. You can do this plot in **SYGRAPH** by using the NOTCH option.

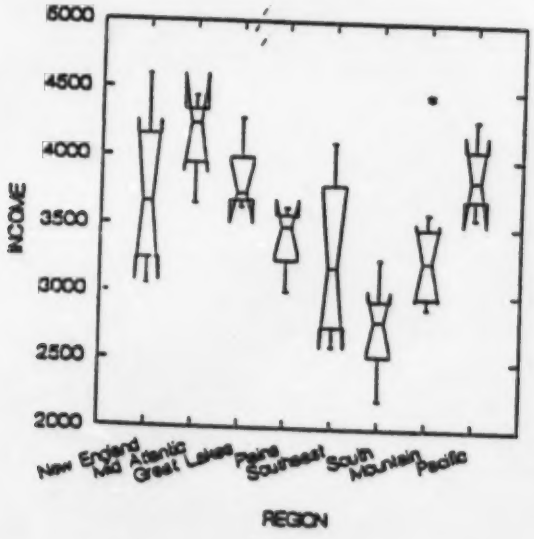
Example 8.3 shows a notched box plot of INCOME (per-capita income) by REGION for the 1970 US dataset. The boxes are notched at the median and return to full width at the lower and upper confidence interval values. Notice that some of the outer confidence limits extend beyond the hinges (the horizontal lines on either side of the narrow median line). This is unaesthetic but adheres to Tukey and McGill's original standard for the plot.

Notches are especially handy for judging differences between groups. Note, for example, that the South had a significantly lower income than New England, but not significantly lower than the Mountain states.

8.3
Notched box plot

Here is a notched plot of INCOME by REGIONS.

```
BOX INCOME*REGIONS / NOTCH.NSORT
```



Appendix 9

Protocol for Determining Live/Dead Zebra Mussels Using Electric Shock

1. With the power off, connect the high voltage function generator to the electrode cell. Clip one lead to the outer ring and the other lead to the centre pin. The capacitor discharge device and volt meter can be attached in the same manner.

2. Set the function generator to sine wave.

3. Fill the cell three quarters full with dechlorinated tap water ($\sim$355 $\mu S\ cm^{-1}$).

4. Transfer mussels post-experiment from test tank or control tank into individual petri-dishes containing water.

5. Observe mussels in petri-dish, tap any open mussel; if it closes, count as live and remove.

6. No response in open mussel, place in electrode cell, posterior end of mussel to the centre pin. Discharge the capacitor discharge device (or HVFG) while observing the mussel for a response. A response can be valve closure or tissue movement. If there is no response then the mussel is dead.

7. Minimally stressed control mussels will generally open first so leave these to the end. Any which are incidentally observed opening during the testing of post-experiment mussels can be removed and counted as live.

8. Repeat steps 5 through 7 and change water in cell frequently to avoid water temperature and turbidity increase.

9. If mussel remains closed in sample dish or after handling, place in electrode cell, set the frequency dial to 1 and the voltage dial to zero. While carefully observing the mussel at the posterior edge for movement (tissue or valve opening) increase the voltage. The tissue may move or the valves open and close or the foot may move.

   If the above does not induce movement, set the voltage dial to 0.5 and the frequency dial to zero and repeat the above steps while varying the frequency.

10. If no movement can be determined by above methods, or if inconclusive* results have been obtained, then one of the mussel valves is removed and voltage is again applied using the high voltage function generator or capacitor discharge device. Observe exposed mussel tissue for movement.

    *NB Air bubble expansion can cause a dead mussel to partially open, therefore rechecking inconclusive results by this method will confirm whether the mussel is live or dead.

11. **Safety - use only plastic probes to move a mussel in a charged cell. Turn off the devices when removing a mussel or placing a mussel in the cell. Turn off devices to change the water.**